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Studies on C-phycocyanin from *Cyanidium caldarium*, a eukaryote at the extremes of habitat

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Abstract

C-Phycocyanin, a biliprotein, was purified from the red alga, *Cyanidium caldarium*. This alga grows at temperatures up to 57°C, a very high temperature for a eukaryote, and at pH values down to 0.05. Using the chromophores on C-phycocyanin as naturally occurring reporter groups, the effects of temperature on the stability of the protein were studied by circular dichroism and absorption spectroscopy. The protein was unchanged from 10 to 50°C, which indicates that higher temperatures are not required to cause the protein to be photosynthetically active. At 60 and 65°C, which are above the temperatures at which the alga can survive, the protein undergoes irreversible denaturation. Gel-filtration column chromatography demonstrated that the irreversibility is caused by the dissociation of the trimeric protein to its constitutive polypeptides. Upon cooling, the α and β polypeptides did not reassemble to the trimer. Unlike phycocyanins 645 and 612, the C-phycocyanin does not show a reversible conformational change at moderately high temperatures. At constant temperature, the C-phycocyanin was more stable than a mesophilic counterpart. It is designated a temperature-resistant protein.

Keywords: High temperature eukaryote; Phycocyanin; Biliprotein; *Cyanidium caldarium*

1. Introduction

The earth may be understood as a planet where a multiplicity of life fills a vast array of habitats. Prokaryotes and Archea are considered to be able to live in the most difficult environments. There are, however, situations where eukaryotes, and even photosynthetic eukaryotes, occupy extraordinarily hostile habitats. *Cyanidium caldarium*, a truly unusual life-form, thrives in acidic hot springs. Growth conditions have pH values down to 0.05 and temperatures up to 57°C [1,2]. It is eukaryotic and is classified as an atypical red alga. The red algae are photosynthetic and have chlorophyll *a* and biliproteins, as does *C. caldarium.*

C. caldarium was first isolated for scientific study by Allen [3] from 'Lemonade Spring', The Geysers in Sonoma County, California. There are several geothermal areas in Yellowstone National Park and others throughout the world that support *C. caldarium* [1,3]. Very few eukaryotes can actively grow at 57°C or higher. This alga provides an important op-

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[1] Recently, Dr. Mercedes R. Edwards passed away. She will be greatly missed as a colleague and a wonderful friend.

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portunity to study a macromolecule from a eukaryote that thrives at a relatively high temperature. *C. caldarium* is, however, not a thermophile and its optimum growth temperature has been determined to be 45°C [1].

To exemplify the salient ranges in ecosystems that the eukaryotes are capable of occupying, a unique biliprotein has been isolated from a red alga growing under thick sea ice in the antarctic region [4]. Instead of the high temperatures and acidity in which *C. caldarium* thrives, this antarctic alga, as well as others, prospers at −2°C in varying conditions of darkness and dim light.

Biliproteins are light harvesting photosynthetic pigments found in cyanobacteria, red algae, and cryptomonads. They absorb solar radiation in spectral regions where chlorophyll has low absorptivity. Energy migrates through the open-chain tetrapyrrole chromophores of the biliproteins until it reaches the chlorophyll *a* within the thylakoid membrane. In cyanobacteria and red algae, the biliproteins are organized into phycobilisomes. In *C. caldarium*, the biliproteins are C-phycocyanin and allophycocyanin [5–10]. It is of particular interest that the amino acid sequence of this C-phycocyanin closely resembles that for cyanobacterial C-phycocyanins [9–11]. The alga was shown to have phycobilisomes [12]. The crystal structure of C-phcocyanin from *C. caldarium* has been obtained [13]. Its structure has produced new insights into energy migration through the rods of the phycobilisomes

There is no published study of the light-harvesting properties for C-phycocyanin from *C. caldarium* as a function of temperature. In this research, bilins, which are covalently attached to apoprotein, were used as naturally occurring reporter groups to monitor the effects of temperature. Additionally, gel-filtration column chromatography was employed to examine the assembly state of the C-phycocyanin. C-Phycocyanin is located in the rods of the phycobilisomes. When extracted into dilute buffer, the phycobilisomes dissociate, and the C-phycocyanin is found as trimers ($\alpha_3\beta_3$), hexamers, and stacks of hexamers. Circular dichroism (CD) and UV/VIS absorption spectroscopy together with gel-filtration column chromatography were employed to determine the reversibility or irreversibility of the temperature-induced changes in the protein.

There are studies on the behavior of C-phycocyanin from a cyanobacterial thermophile, *Synechococcus lividus*, strain I. This alga grows at temperatures up to 73°C. Its purified C-phycocyanin is both highly aggregated and has identical spectroscopic behaviors at 20 and 70°C [14,15]. We call this behavior 'temperature resistant'. The C-phycocyanin from another cyanobacterium *S. lividus*, strain III, which grows up to 55°C, is quite different. The purified protein is highly assembled at 50°C and dissociated markedly at 20°C [16,17]. The dissociated protein at 20°C retains its ordered state, however, and is not denatured as determined by its absorption spectrum [15]. We call this a 'cold-dissociated' protein. Other proteins have been shown to cold denature ([18] and references therein), but C-phycocyanin from *S. lividus*, strain III, dissociates, but does not otherwise denature at 20°C. Other C-phycocyanins have been studied as a function of temperature [19,20], and C-phycocyanin from *C. caldarium* has been studied using urea as a denaturant [21].

The properties of biliproteins have been reviewed [22–29]. A major question in considering C-phycocyanin and allophycocyanin is the interaction between the two closest bilins in the trimeric oligomers, located at α84 and β84 on adjacent polypeptides [29]. There is a difference in viewpoint on the relationship between these two phycocyanobilins on allophycocyanin trimers. One set of results appears to suggest that the two bilins are localized entities and energy is transferred between them by Förster resonance energy transfer [30–34]. The second hypothesis suggests that the two bilins are close enough and at a proper orientation so that they undergo exciton coupling and energy is delocalized and shared by the bilin pair [35–43]. For these allophycocyanin trimers, the two visible bands observed in the absorption spectrum are either the two separate bilins in the Förster resonance case, or the upper and lower exciton bands in the case of exciton splitting. Between two pairs of bilins, energy would be transferred by the Förster resonance mechanism. For C-phycocyanin trimers, there is much less evidence to support exciton coupling between these same two bilins, but some data also points in this direction [44]. Other energy transfer events have recently been studied for C-phycocyanin hexamers [45]. Methods to dissociate trimers of allophycocyanin and C-phycocyanin

to monomers (αβ) were important in these studies [46–48].

There have been many thermophilic and mesophilic proteins studied in order to understand the basis for thermostability in proteins (e.g., [49–51]). Beadle et al. [49] have reviewed some of this literature, and they discussed the difficulties in finding unambiguous answers to the question of what makes a protein stable to high temperature.

C. caldarium can grow in the laboratory in 1 N H_2S0_4. In soil of Yellowstone National Park, Brock [1] reported *C. caldarium* commonly growing at pH 0.05. However, the interior of the cells is at neutral pH [3]. An important benefit from growth at such highly acidic conditions could be the lack of competition from other photosynthetic organisms.

2. Materials and methods

C. caldarium was isolated from an acid thermal area of Yellowstone National Park by Dr. W.N. Doemel, who generously donated a culture to Dr. Edwards. Mercedes Edwards grew axenic cultures in Albany in Ascione's medium [52] at pH 2–3 and 50–54°C. A florence flask containing 10 l of medium was innoculated with a dense suspension of cells to give a final concentration of 1×10^6–5×10^6 cells/ml. The culture was agitated with a plastic-encased magnet driven by a magnetic stirrer and by N_2 and CO_2 (95:5 ratio) (Matheson, Rutherford, NJ, USA), which was bubbled into the flask at about 3 ml/min. The culture was illuminated with daylight fluorescent bulbs and tungsten lamps at 2200–5400 1×. Cells were generally harvested after 10–15 days in the late-exponential stage of growth. In some cases, where harvesting was done after only 5–6 days, the results did not show any detectable difference in the characteristics or the relative quantity of the C-phycocyanin.

Algal cells were collected by centrifugation and disrupted by a Braun mechanical cell homogenizer, model MSK (Bronwill Scientific, Rochester, NY, USA). About 6–8 g of cells were suspended in 30 ml of sodium phosphate buffer, pH 6.0, ionic strength (*I*) 0.1, and transferred to a 60 ml Braun Melsungen glass flask containing 6–8 g of glass beads in a mixture of sizes (0.25, 0.45 and 1.0 mm diameter). The homogenizer was chilled, and cell disintegration was accomplished in periods of 1–2 min for a total to 10–15 min. The supernatants were separated from the glass beads and cell membranes by repeated centrifugation at 30-min intervals for a total of 4–6 h in the SS34 rotor of an RC-2B Sorvall centrifuge at $18\,000\times g$ at about 4°C. The supernatant was treated with 2% (w/v) protamine sulfate solution for 30 min and centrifuged at $18\,50\times g$ for 15 min. The supernatant solution was then adjusted to 50% (w/v) saturation with $(NH_4)_2SO_4$ at pH 6.0, and the C-phycocyanin was precipitated. The C-phycocyanin fraction was then dialyzed repeatedly into 30%-saturated $(NH_4)_2SO_4$ in sodium phosphate buffer, pH 6.0, followed by extensive centrifugation at $20\,000\times g$ at 30-min intervals for a total of 3 h or more. For many of the samples, it was necessary to use sucrose gradients to separate allophycocyanin from the C-phycocyanin (Edwards, unpublished results). This procedure was continued until the A_{620}/A_{280} ratio of C-phycocyanin in phosphate buffer was 4.0 or greater. Purification by the same procedure and excluding the protamine sulfate treatment yielded an identical phycocyanin, but its addition improved the process significantly.

Phycocyanins 612 and 645 were isolated from laboratory harvests of the cryptomonads *Hemiselmis verescens* and *Chroomonas* sp., respectively, by freeze/thawing the cells into pH 6.0 buffer. The proteins were purified as described previously [53].

C-Phycocyanin from the mesophilic cyanobacterium, *Phormidium luridum*, was purified from laboratory harvests. Cells were lysed into pH 6.0 buffer using lysozyme. The purification was carried out using ammonium sulfate precipitation and hydroxylapatite column chromatography.

CD was obtained on a JASCO J-720 spectropolarimeter. A 5-mm light path was used. The temperature of the samples was maintained by water circulating from a Neslab RTE-111 circulating bath. The temperature of the bath was controlled from a remote sensor located very near the sample cuvette. Samples were kept at each temperature for 10 min before a spectrum was recorded. UV/VIS spectra were taken on a Beckman DU-640 spectrophotometer, and a Peltier device controlled the temperature. The Peltier system provided faster heating and cooling than the water circulator. CD in the ultra-violet

was carried out from 180 to 260 nm at 20°C. The secondary structures were calculated by the Selcon method [54].

Gel-filtration column chromatography was carried out using a Waters 625 liquid chromatography system. Samples were injected from an autosampler (Waters 717 plus) onto a Shodex KW-803 column (Waters). The column (8×300 mm) was equilibrated in buffer and run under isocratic conditions with the temperature maintained by a column heater. A photodiode array detector (Waters 996) was used to obtain the spectra of individual bands.

The concentration of C-phycocyanin used in these various experiments was 0.10 g/l, and except when otherwise indicated the experiments were carried out in pH 6.0, 0.1 *I*, sodium phosphate buffer. For phycocyanins 645 and 612, the concentration was 0.15 g/l in pH 6.0 buffer.

3. Results and discussion

CD spectroscopy in the energy region of the absorption of the bilins was used to study the effects of temperature on the properties of C-phycocyanin from *C. caldarium* (Fig. 1). From 10°C to a typical growth temperature of 50°C, there was no change in the CD spectra of the protein. At 60 and 65°C, there were noticeable changes in the spectra (Fig. 1). The changes were irreversible upon cooling.

The UV/VIS absorption spectra were also examined as a function of temperature (Fig. 2), and it was observed that at 65°C there was a major change from a native to a denatured spectrum. In native C-phycocyanin, the bilins are maintained by the apoprotein in an extended configuration, while in disordered protein the bilins become more cyclic [29]. The cyclic state gives the bilins a lower visible and a higher near-UV absorbance as was observed at 65°C for the C-phycocyanin (Fig. 2). The samples were cooled from 60°C or 65°C back to 20°C, and the results show that the changes in the protein were not completely reversible (Fig. 2). Even when heated to 55°C, a very small amount of irreversibility was noted (data not shown) upon cooling.

The optical results as a function of temperature are shown (Fig. 3), and the stability of the protein up to its physiological growth temperature is manifest. The denaturation of the protein above 55°C suggests that this is one possible factor concerning why the alga cannot grow above 57°C. A comparison was made to the thermal denaturation of C-phycocyanin from the mesophile, *P. luridum*. Using CD and protein in the same concentration, buffer, and cuvette as used for the protein from *C. caldarium*, the C-phycocyanin from *P. luridum* was found to show changes in spectra at lower temperatures than the protein from *C. caldarium*. A 50% change in the CD from that at 20°C was achieved for the protein from *P. luridum* at a 10°C lower temperature (data not shown).

The question arises, why the changes observed by visible CD were irreversible even at the lowest temperature that showed a change. To examine this oc-

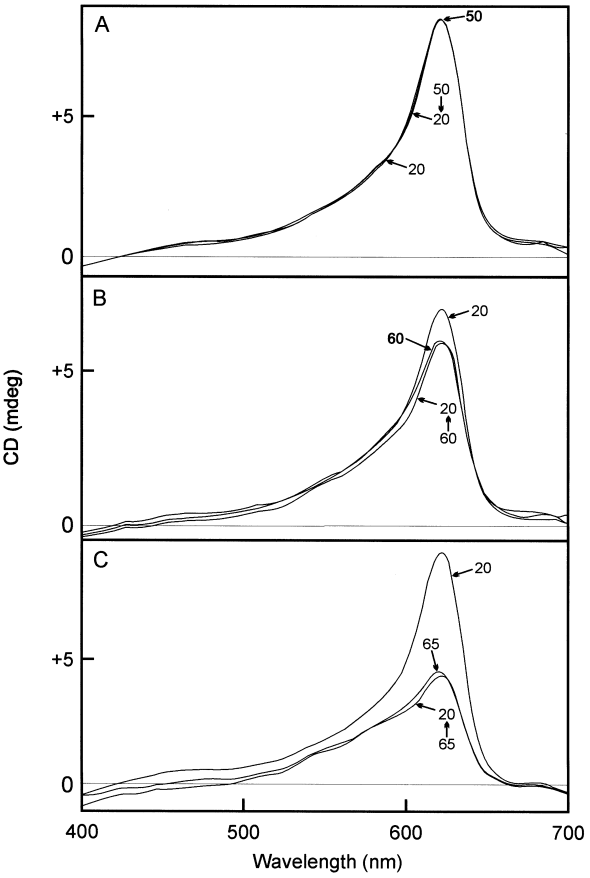

Fig. 1. CD spectra of C-phycocyanin from *C. caldarium* at various temperatures. Protein was at 0.1 g/l in pH 6.0 buffer. A 5-mm light path was used. In each panel, the spectra at 20°C, at a higher temperature, and when returned to the lower temperature are given. The numbers on the curves are the temperatures.

currence, gel-filtration column chromatography was carried out on the protein as a function of temperature (Fig. 4). At 24°C, the solution of C-phycocyanin at pH 6.0 was observed to be a mixture of trimers and a lesser amount of hexamers. Both of these elution bands showed a typical C-phycocyanin spectrum using a photodiode array detector (data not shown). These assembly states were determined from the estimated molecular masses determined by running materials of known molecular mass through the column immediately prior to running the C-phycocyanin. A calibration curve was generated and the unknown molecular masses calculated. The molecular mass of a trimer was expected to be about 106 000, and that of a hexamer was expected to be double that plus the molecular mass of a linker over 30 000, totaling about 242 000. Linkers serve to stabilize the assembly of hexamers and larger rod assemblies. The measured molecular masses were estimated at 104 900 for the predominate band and 289 500 for the less prominent band.

In the exact temperature range where changes were observed in the optical spectra (Fig. 1), the gel-filtration elution profiles showed a new band at lower molecular masses, between 11 000 and 17 000 that is not present at 24°C (Fig. 4B). This range of molecular masses is suggestive of the individual polypeptides (α and β), which comprise C-phycocyanin. When the solutions at high temperature were cooled to 24°C, there was no reassembly observed for the low molecular mass band (Fig. 4C). Reversibility of oligomer formation was looked at periodically over a period of time, but no reassociation was observed (data not shown). The irreversibility in the gel-filtration experiments corresponds to the results using optical spectroscopy. It appears that once formed in these high-temperature experiments, these polypeptides when cooled do not reassociate to form the native forms of C-phycocyanin. This property may perhaps be related to the tendencies of these polypeptides, α and β, to self dimerize [55]. In other studies, dimerization was not observed for one of these peptides [56,57]. The large amount of separated polypeptides at 65°C corresponded nicely to the extensive changes in visible CD and UV/VIS absorption at that temperature.

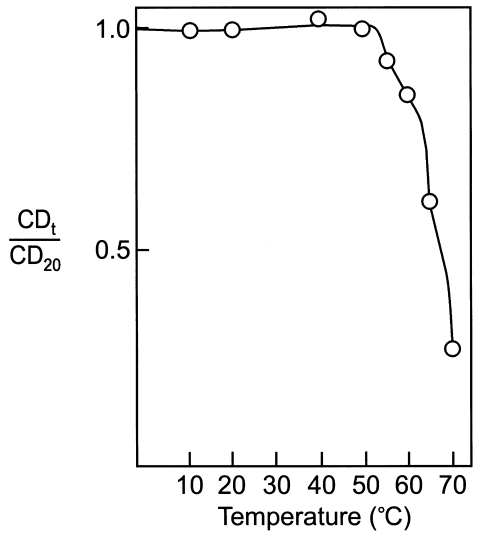

Fig. 3. The effects of temperature on the CD maximum of C-phycocyanin from *C. caldarium*. The protein concentration was 0.1 g/l in pH 6.0 buffer. A 5-mm light path was used.

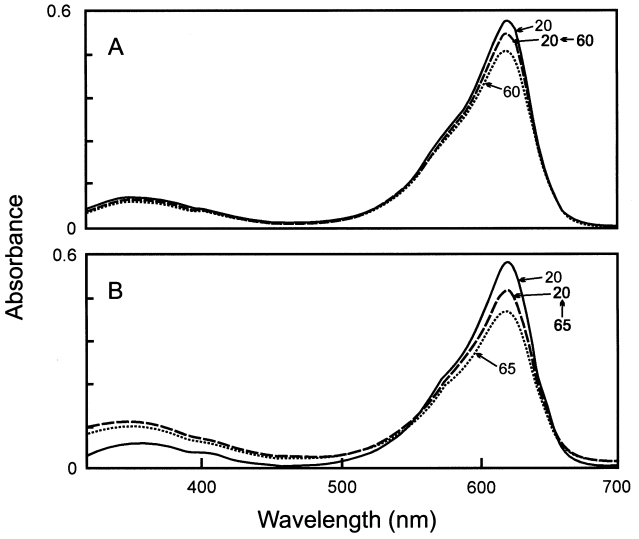

Fig. 2. UV/VIS absorption spectra of C-phycocyanin from *C. caldarium* as a function of temperature. The numbers on each spectrum are the temperatures. The protein was at 0.1 g/l in pH 6.0 buffer.

For phycocyanin 645 isolated from a cryptomonad, a reversible change in the visible CD was observed at 45°C (Fig. 5A). The blue edge of the spectrum showed a particularly large reversible change at this temperature (Fig. 5B). A small negative band present at 20°C is absent at 45°C, and a return to lower temperature totally restores it. This result would not have been detected using UV/VIS absorption spectroscopy and points to the utility of CD in

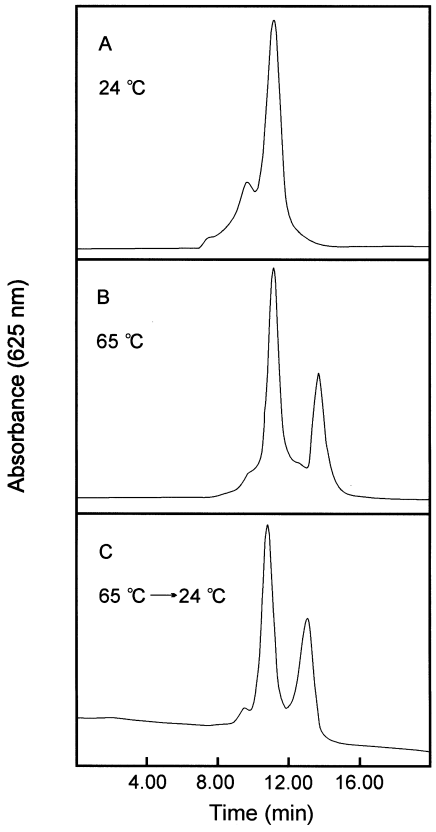

Fig. 4. Gel-filtration column chromatography of C-phycocyanin from *C. caldarium* as a function of temperature. A is the elution profile at ambient temperature, B is the elution profile at 65°C; and C is the elution profile of a sample heated to 65°C and the cooled to ambient temperature. There is a low molecular mass band not found at ambient temperatures. The protein was 0.1 g/l before injection onto the column.

the visible region as an important tool in the study of chromoproteins. Using another cryptomonad biliprotein, phycocyanin 612, a temperature-induced reversible change in the CD was also observed at 40°C (Fig. 5C). For both of these cryptomonad biliproteins, the spectral changes were caused by changes in the tertiary structure of the protein, and the secondary and quaternary structures remained unchanged at the elevated temperatures [58,59]. Using CD, a similar reversible change was not obtained for the C-phycocyanin (data not shown).

It was of interest to examine the stability of C-phycocyanin from *C. caldarium* at a fixed temperature. The effect of pH on the aggregation state of the protein was examined by gel-filtration column chromatography (Fig. 6). C-Phycocyanins from certain mesophilic cyanobacteria have been shown to dissociate to homogeneous solutions of monomers ($\alpha\beta$) at pH 3.9 [60]. This C-phycocyanin was treated at a series of pH values from 3.9 to 3.0, and gel-filtration experiments were performed. Down to pH 3.2, some trimers were still observed (Fig. 6), and only at pH 3.0 were the trimers nearly fully dissociated. This result suggests that the trimers of C-phycocyanin from *C. caldarium*, even at a low temperature, 23°C, had an augmented stabilization when compared to certain other C-phycocyanins. Another aspect of this experiment was that instead of obtaining monomers, these trimers completely dissociated to the individual polypeptides as suggested by the molecular masses that were obtained.

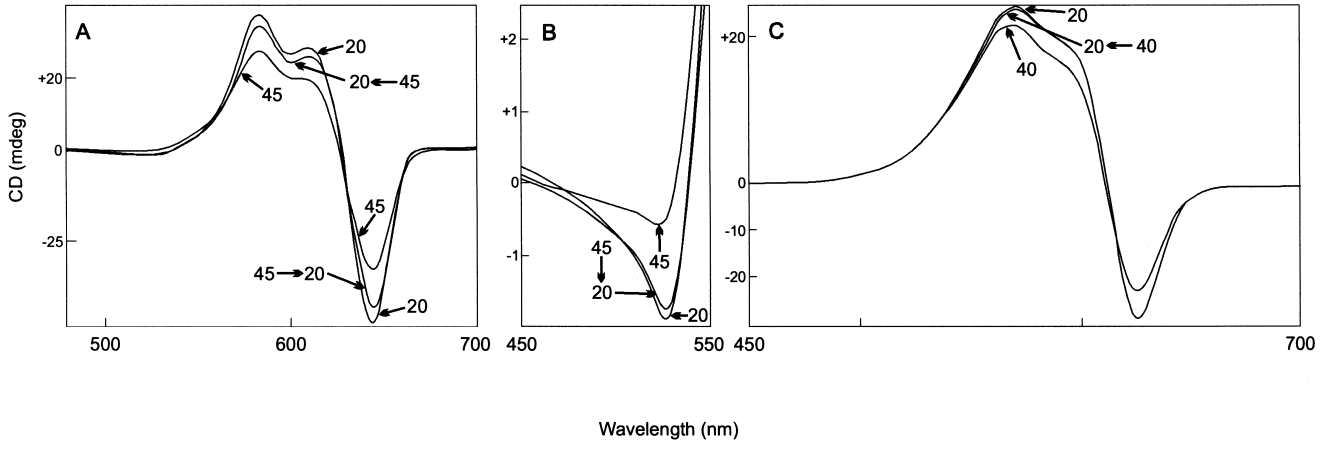

Fig. 5. CD spectra of phycocyanin 645 and 612 as a function of temperature. A and B are phycocyanin 645, and C is phycocyanin 612. Temperatures are indicated on each spectrum. Proteins were at 0.15 g/l in pH 6.0 buffer. A 5-mm light path was used.

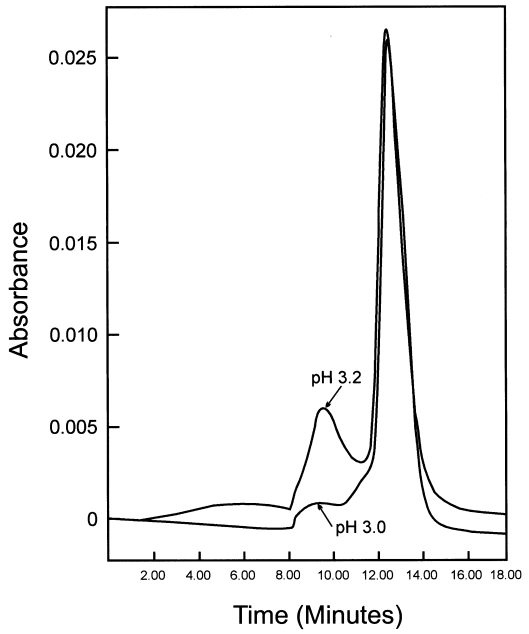

Fig. 6. Gel-filtration column chromatography studies on C-phycocyanin from *C. caldarium* at pH 3.2 and 3.0. The protein concentration was 0.1 g/l before injection onto the column.

Kao et al. [5] have shown that C-phycocyanin from *C. caldarium* is not a 'cold-dissociated' protein, from an analysis of the high assembly state of the protein at low temperatures. The current studies on its CD spectra show that it is functionally equivalent from 10°C to a physiological temperature, 50°C (Figs. 1 and 3). Its CD spectra remains unchanged over this entire temperature range. These findings suggest that the protein required no heat-induced properties to perform its functions in photosynthesis. This constancy of spectra is observed at moderate temperatures where for certain other biliproteins reversible changes are observed (Fig. 5). The protein fits the characteristics of a 'temperature-resistant' protein, as observed with the C-phycocyanin from *S. lividus*, strain I [14,15]. Its hallmark for stability is to remain inflexible toward structural change over a wide range of temperatures. No stable conformational intermediate is observed between the trimer and the disordered individual polypeptides. The protein denatures irreversibly at the temperature at which the alga is no longer viable (Fig. 4).

CD in the ultra-violet region from 260 to 180 nm has been used to compare the secondary structures of C-phycocyanin from *C. caldarium* and *P. luridum* (data not shown). At 20°C, the red algal protein had $47.5 \pm 2.2\%$ α-helix, $14.6 \pm 4.5\%$ β-sheet, $20.3 \pm 1.8\%$ β-turn, and $17.3 \pm 3.2\%$ other. The cyanobacterial protein possessed $67.1 \pm 8.9\%$ α-helix, $5.7 \pm 0.9\%$ β-sheet, $14.6 \pm 3.4\%$ β-turn, and $14.8 \pm 3.0\%$ other. Surprisingly, the red algal protein had significantly less α-helix and more β-sheet than the other C-phycocyanin. Both, however, can be considered predominantly α-helical in their secondary structures. When the other data obtained for the red algal protein is considered it is clear that the lower α-helix content is probably not the result of denaturation. It would be of interest to study the denaturation of this C-phycocyanin as a function of temperature using CD in the UV, but the complexity of many possible changes in structure, including aggregation and dissociation, transitions among secondary structures, tertiary structure changes, and separation of the two subunits, made this a difficult approach for analysis. The use of bilins as reporter groups for thermal transitions, although limited to input from restricted regions of the protein, permitted a more straightforward analysis.

In very early studies on thermophilic cyanobacteria from Yellowstone National Park, Brock [61] noted that photosynthetic life had an upper temperature for existence at 73°C, while non-photosynthetic organisms could grow at much higher temperatures. Brock [61] proposed that possibly some part of the photosynthetic system accounted for the temperature limitation. For C-phycocyanin from *C. caldarium*, the proposal seems very warranted based on the results obtained for purified protein (Fig. 3). The upper temperature limit for growth of the alga corresponds very nicely with the range of thermal stability of this particular part of the photosynthetic apparatus. Miller et al. [62] have discussed how the upper limit for temperature of thermophilic cyanobacteria may be extended because of the lack of competition from other photosynthetic organisms in these habitats. This proposal would appear to apply to the red alga, *C. caldarium*, as well, but finally the thermal stability of the macromolecule determines the highest growth temperature.

C-Phycocyanin has also been isolated from another thermophilic cyanobacterium [63].

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